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Biosensor microprobes with integrated microfluidic channels for bi-directional neurochemical interaction

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Abstract

This paper reports on silicon-based microprobes, 8 mm long and 250 μm × 250 μm cross-section, comprising four recessed biosensor microelectrodes (50 μm × 150 μm) per probe shank coated with an enzymatic layer for the selective detection of choline at multiple sites in brain tissue. Integrated in the same probe shank are up to two microfluidic channels for controlled local liquid delivery at a defined distance from the biosensor microelectrodes. State-of-the-art silicon micromachining processing was applied for reproducible fabrication of these experiment-tailored multi-functional probe arrays. Reliable electric and fluidic interconnections to the microprobes are guaranteed by a custom-made holder. The reversible packaging method implemented in this holder significantly reduces cost and assembly time and simplifies storage of the biosensor probes between consecutive experiments. The functionalization of the electrodes is carried out using electrochemically aided adsorption. This spatially controlled deposition technique enables a parallel deposition of membranes and is especially useful when working with microelectrode arrays. The achieved biosensors show adequate characteristics to detect choline in physiologically relevant concentrations at sufficient temporal and spatial resolution for brain research. Sensitivity to choline better than 10 pA μM^{-1}, detection limit below 1 μM and response time of 2 s were obtained. The proposed combination of biosensors and microfluidic injectors on the same microprobe allows simultaneous chemical stimulation and recording as demonstrated in an agarose gel-based brain phantom.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

The direct interaction with brain tissue using invasive microprobes gained growing interest in recent years. Together with the increasing performance of recording and stimulating microelectrodes (Wise 2005, Cheung 2007, Normann 2007, Cogan 2008, Valles 2010) as well as biosensors (Fillenz 2005, Wilson and Gifford 2005) and drug delivery systems (Lin and Pisano 1999, McAllister *et al* 2000, Zahn *et al* 2000, Rathnasingham *et al* 2004, Neeves *et al* 2006, Papageorgiou

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et al 2006, Parker *et al* 2007, Foley *et al* 2009) toward more reliable and sensitive measurements, advances are also made in combining some of these functions into a single device (Chen *et al* 1997, Metz *et al* 2004, Johnson *et al* 2008, Seidl *et al* 2010, Frey *et al* 2011). This allows us to (i) record and stimulate in parallel from several electrode sites at high spatial resolution and defined inter-electrode spacing arranged in two- (2D) or three-dimensional (3D) arrays, (ii) increase the number of functionalities per microprobe shank cross-section thus reducing the overall neuronal damage and (iii) perform more complex, multi-parametrical brain studies. Progress was recently achieved in the framework of the European research project *NeuroProbes* (FP6 IST-027017) by developing a platform-based, modular 3D assembly approach of multiple functionalities allowing electrical as well as chemical recording and stimulation (Neves and Ruther 2007, Neves 2007, Ruther *et al* 2008, 2010).

In particular, the combination of biosensors for the detection of neurotransmitters with fluidic microchannels is of high interest as it enables bi-directional chemical interaction with the brain tissue: as an example, the neurochemical impact of fluid stimulation or inactivation of synaptic activity can be monitored simultaneously at the dedicated sites. However, for reliable and spatially resolved experiments this requires small and sensitive biosensors as well as fluidic outlet ports capable of local, low-volume liquid delivery with both system components positioned at well-defined distances on a single device.

Choline—a surrogate marker of freshly released acetylcholine—can be continuously monitored at adequate spatial and temporal resolution using biosensors. Most sensors use the measurement principle of amperometric detection via hydrogen peroxide produced by the oxidase enzyme (Wilson and Gifford 2005). For the deposition of the enzymatic membranes on dedicated metal microelectrodes different methods are used including dip coating (Schuvailo *et al* 2006), drop dispensing (Burmeister *et al* 2002) and electrochemical approaches (Cosnier *et al* 1997). The choice of the applied deposition approach is usually driven by the type of employed microelectrode devices such as conventional wire electrodes, microprobes with multiple integrated microelectrodes or microprobe arrays with a 2D or even 3D microelectrode arrangement.

Following the demand to record in parallel from several electrodes in different brain areas, silicon microtechnology processes are increasingly applied to fabricate multi-electrode arrays (Takeuchi *et al* 2004, Lee *et al* 2004, Musallam *et al* 2007, Aarts *et al* 2008, Hajj-Hassan *et al* 2009, Herwik *et al* 2009, Perlin and Wise 2009, Chen *et al* 2009). The main advantages of these probes are their high design-flexibility as well as precise and reproducible production of arrays comprising multiple microelectrodes per probe shank.

Pressure-driven drug delivery in neuroscience has been combined with microprobe biosensors in the past. It mainly consisted of a manual assembly of stainless steel or glass capillaries with a microprobe (Bruno *et al* 2006, Day *et al* 2006, Parikh *et al* 2008). For these approaches, precise and reproducible relative positioning of the biosensors and the microfluidic components is highly challenging. Further, the injection of several liquids with such a device is limited as well. In addition, the non-parallel alignment of microprobe and capillary during assembly can result in increased brain damage and shank distortion during implantation. This issue has been addressed using microfabricated injectors aligned in parallel (Frey *et al* 2011) or by integrating microchannels directly into the microprobe shanks. The latter has been presented by different groups using silicon substrates (Chen *et al* 1997, Cheung *et al* 2003, Seidl *et al* 2010), glass (Lin *et al* 2009, Vrouwe *et al* 2008) or flexible polymers (Metz *et al* 2004, Moon *et al* 2006, Ziegler *et al* 2006, Fernandez *et al* 2009), predominantly for microprobes comprising bare metal electrodes for electrophysiology applications.

Integrating microfluidic channels into biosensor microprobes is more complicated as the biosensors require the deposition of one or more polymeric membranes (enzymatic membrane, anti-interference layer). This coating has to be spatially controlled to enable a highly parallel modification of designated electrodes only. Contamination of other electrodes or clogging of the microfluidic channels by exposure to the membrane cocktail needs to be avoided.

When multiple functions are integrated in microdevices, connectivity becomes challenging: fluidic and electrical contacts have to be ensured under tight spatial constraints. Additionally, packaging is known to be a major cost factor in the semiconductor industry as well as in the field of medical microdevices. Therefore simple, robust and re-usable set-ups are highly desirable, especially when parts of the device—as for example biosensors—are characterized by a relatively short lifetime and thus life-limiting components of the neural system.

In this contribution, we report on multi-shank silicon microprobes with several recessed microelectrodes and microfluidic channels integrated in the same probe shank. The microelectrodes are coated with an enzyme layer using electrochemically aided adsorption and an anti-interference layer by electrochemical polymerization. Both layers are tailored to the requested selective detection of choline at concentrations below 10 μM. The symmetric layout of the microprobes is specifically designed for simultaneous measurements in both hemispheres of the prefrontal cortex of the rat's brain. The microprobes are reversibly packaged into a small, custom-made device providing electrical as well as fluidic connections.

2. Materials and methods

2.1. Microprobe layout and fabrication

The microprobe type presented in this paper was fabricated within the FP6 European project *NeuroProbes* (IST 027017). The selected approach is fully compatible with the modular concept pursued by the consortium for assembly of multifunctional microprobes into a standardized platform (Aarts *et al* 2008, Herwik *et al* 2009, Neves and Ruther 2007). The geometrical layout and fabrication process of the microprobes are based on the work described by Seidl

et al (2010). In the present contribution, the design of the recessed electrodes and corresponding interconnecting leads were adapted with respect to the specific requirements of the choline biosensors and the assembly of the probes in a re-usable package.

The layout of the microprobe is shown in figure 1. The design consists of two parallel probe shanks with a length of 8 mm and cross-section of 250 μm × 250 μm (height × width). The two shanks have a pitch of 1.5 mm. The probe dimensions were chosen to determine choline concentrations and provide fluid supply in both hemispheres of the prefrontal cortex of the rat's brain. Each shank is connected to a common rectangular probe base and comprises either one or two fluidic microchannels with a cross-section of 50 μm × 50 μm. The outlet ports have a diameter of 25 μm and are centered on the topside of the probe shank at a distance of 1.5 mm from the microprobe tip. In the case of two channels per probe shank, outlet ports are positioned next to each other with a spacing of 100 μm. The inlet ports of the microchannels are provided from the rear side of the microprobe base. The base has a size of 4 mm × 6 mm and a thickness of 400 μm, which is convenient for probe handling. Each shank comprises five platinum (Pt) microelectrodes, four of them being placed around the fluidic outlet ports and separated by 200 μm. These electrodes have a size of 50 μm × 150 μm and are placed in a 10 μm deep recess to protect the biosensitive membrane that is subsequently immobilized on the electrodes. The fifth electrode with a size of 80 μm × 1200 μm is not recessed and integrated in the middle of the probe shank. It can be used as a pseudo-reference or counter electrode for the electrochemical detection configuration. For the first, Ag/AgCl can be electrochemically deposited on the Pt surface. Especially, for *in vivo* application this may be more practical. All electrodes are addressed by ten connection pads (0.3 mm × 1.4 mm in size) integrated on the probe base topside.

The microprobe fabrication is based on a process as described in detail elsewhere (Seidl *et al* 2010) and only briefly repeated here. Main focus lies on the adapted fabrication steps for the biosensor application. All selected technologies are compatible with the process technology used within the *NeuroProbes* project to realize recording probe arrays (Herwik *et al* 2009).

The fabrication process applies standard 4 inch, 300 μm thick, double-side-polished (100) silicon wafers. In a first step, the channel structures and trenches defining the probe geometry are etched using a two-stage deep reactive ion etching (DRIE) process using silicon oxide and photoresist as masking layers. The channels are then oxidized and covered by a second oxidized silicon wafer using direct wafer bonding and annealing at 1050 °C. This process creates the buried channels of the neural probes. Subsequently, the cover wafer is ground and polished to a thickness of 100 μm at a surface roughness of less than 14 nm using a commercial grinding process.

At this point, the fabrication process differs from Seidl *et al* (2010): A thermally grown oxide (200 nm) is used as masking layer to etch the 10 μm deep electrode recesses using

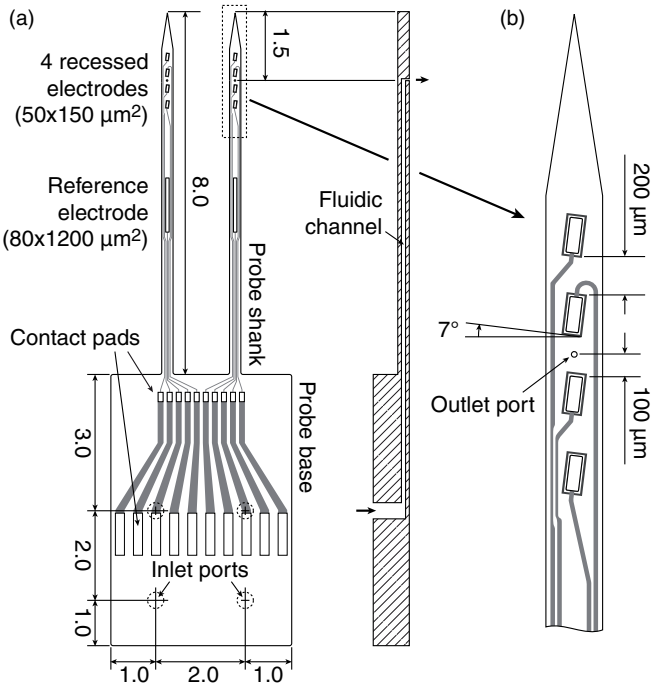

Figure 1. Microprobe layout. (a) Top and side view of the microprobe comprising four recessed electrodes, one reference electrode and one fluidic microchannel per probe shank (relevant dimensions are given in mm). (b) Enlarged view of the microprobe tip illustrating the four recessed electrodes with respective interconnecting leads and the position of the fluidic outlet.

KOH (potassium hydroxide, 40 wt%, 60 °C). As the probe layout is rotated by 7° with respect to the (110) direction of silicon in order to improve the mechanical stability of the wafer stack during processing (Seidl *et al* 2010), the recesses and electrodes have to be rotated back by −7° to match the recess edges to the (110) direction and thus obtain well-defined inclined sidewalls after the KOH etch. These sidewalls are required to guide the metal interconnections and overcome the 10 μm step between the recess—in which the electrode is placed—and the top surface of the probe shank. The silicon substrate is then insulated by a stack of thermal silicon oxide and LPCVD (low pressure chemical vapor deposition) silicon nitride with a thickness of 200 nm each. The metallization, i.e. 20 nm of tantalum (Ta) serving as the adhesion promoter and 130 nm of Pt deposited using e-beam evaporation, is patterned by an adapted lift-off process using a combination of the thick photoresist AZ4562 (AZ Electronic Materials GmbH, Wiesbaden, Germany) and LOR 3B (MicroChem Corp., Newton, USA). A second 200 nm thick LPCVD silicon nitride layer and 200 nm thick PECVD (plasma enhanced CVD) oxide layer are used as top passivation which is opened at the electrode sites and connection pads by a combined dry and wet etching process, i.e. reactive ion etching (RIE) and an H_3PO_4 etch for 10 min at 160 °C, respectively. Again, thick photoresist is used as a masking layer.

The microprobe patterning follows again the procedure described by Seidl *et al* (2010). First, it applies a 150 μm deep backside DRIE process to define the final shank thickness, the

overall probe geometry as well as the fluidic inlet ports on the backside of the probe base. Second, the dielectric layers on the front side are patterned by RIE followed by a DRIE step with an etch depth equivalent to the thickness of the cover wafer to finally release the microprobes. Small joints retain the microprobes in the remaining wafer grid, where they are kept until use.

2.2. Biosensor functionalization

The detection of choline is achieved by immobilizing the enzyme choline oxidase (ChoOx) on the Pt microelectrode surfaces. ChoOx catalyzes the reaction of choline to hydrogen peroxide (H_2O_2) that can be electrochemically oxidized at a potential of 0.7 V versus Ag/AgCl. As other electro-active molecules are present in the extracellular fluid of the brain at relatively high concentrations, special precautions have to be taken to ensure selective measurements. Furthermore, adapted deposition methods have to be chosen for these enzyme layers as the applied microprobe arrays comprise multiple electrodes arranged in up to three dimensions as well as microfluidic channels. These deposition methods have to ensure spatial control and prevent cross-contamination, e.g. due to unspecific adsorption on other electrodes or clogging of the integrated microchannels. Electrochemical deposition methods offer these advantages and allow parallel deposition on several dedicated electrodes.

The enzymatic membranes are deposited using electrochemically aided adsorption. This method has been used before for microprobe arrays without microchannels (Frey *et al* 2010). Briefly, the probes are immersed into a solution containing the enzyme choline oxidase (ChoOx), bovine serum albumin (BSA) and the cross-linking agent glutaraldehyde (GA), all from Sigma-Aldrich, Buchs, Switzerland. The application of a positive potential train on chosen electrodes generates an electrophoretic movement of the negatively charged proteins toward the electrode. The local rise in concentration initiates a cross-linking and immobilization of the enzyme on the corresponding electrode.

In a second step, *m*-phenylenediamine (*m*-PD, Sigma-Aldrich) is electrochemically polymerized on the surface of the electrode using cyclic voltammetry (0.0–0.9 V versus Ag/AgCl, 50 mV s^{-1}). This generates a semi-permeable membrane that rejects certain electro-oxidizable species such as ascorbic acid and dopamine that can interfere with the biosensor signal.

Additionally, adjacent electrodes can be covered with a protein membrane in the same way as described above, however without the enzyme ChoOx, i.e. comprising BSA and GA only. The reason for this is that these membrane-covered electrodes can be used as integrated negative control and in a differential measurement configuration to filter out external influences on the signal (see below). This non-active membranes are required to match diffusion properties with the active, enzyme-coated electrodes. The *m*-polyphenylenediamine (*m*-PPD) is applied on these electrodes as well. The functionalized microprobes are stored in phosphate buffered saline (PBS) at 4 °C.

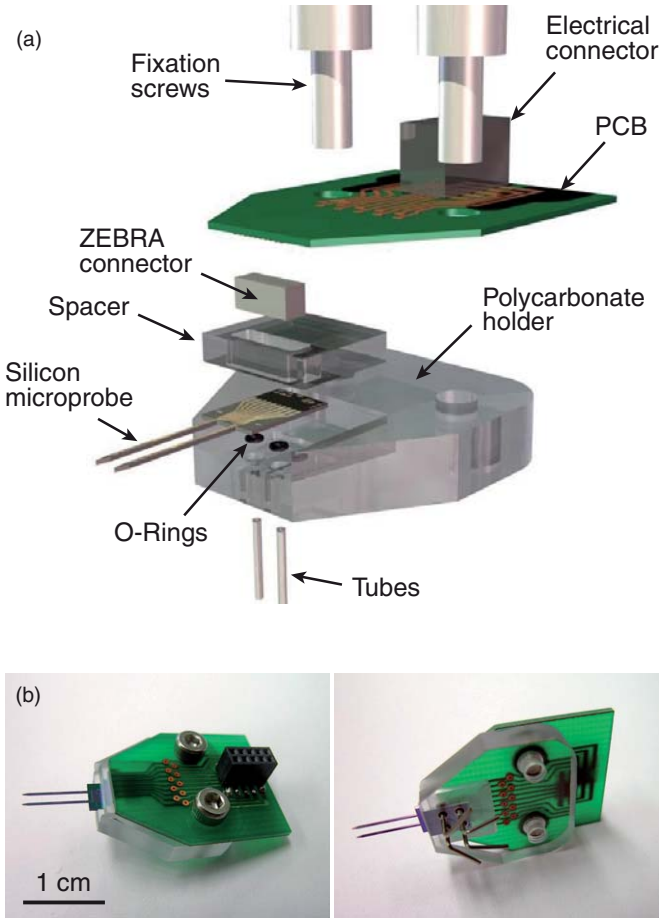

Figure 2. Reversible packaging of the microprobe. (a) Exploded view of the different components: The microprobe is clamped between o-rings providing a tight fluidic connection from the bottom and a ZEBRA® connector squeezed between the microprobe pads and the PCB to electrically connect the contact pads on the probe base from the top. (b) Photographs from the top and the bottom of a packaged microprobe.

2.3. Packaging

For packaging the microprobe is clamped between o-rings that provide fluidic connection from the back of the probe base similarly described by Spieth *et al* (2009, 2010) and a ZEBRA® connector (Fujipoly, Nucletron Technologies GmbH, Munich, Germany) from the topside to electrically interface the contact pads, as illustrated in figure 2(a). The main component of this probe package is a custom-made polycarbonate (PC) holder carrying the microprobe. Depending on the number of microchannels in the probes, either two or four o-rings between this holder and the microprobe base dependent on the number of microchannels in the probes are placed into individual cavities to ensure an aligned and leakage-tight connection of the microprobe base to tubes (syringe needle, Microbalance™ 3, 25G 1”—Nr. 18, 0.5 × 25 mm) inserted into the holder. The required clamping force is applied by an intermediate PC spacer, which fits exactly into the holder. The same spacer comprises a slot that aligns the ZEBRA® connector with respect to the connection pads of the microprobe. The thickness of the spacer is defined by the height of the ZEBRA® connector minus the required

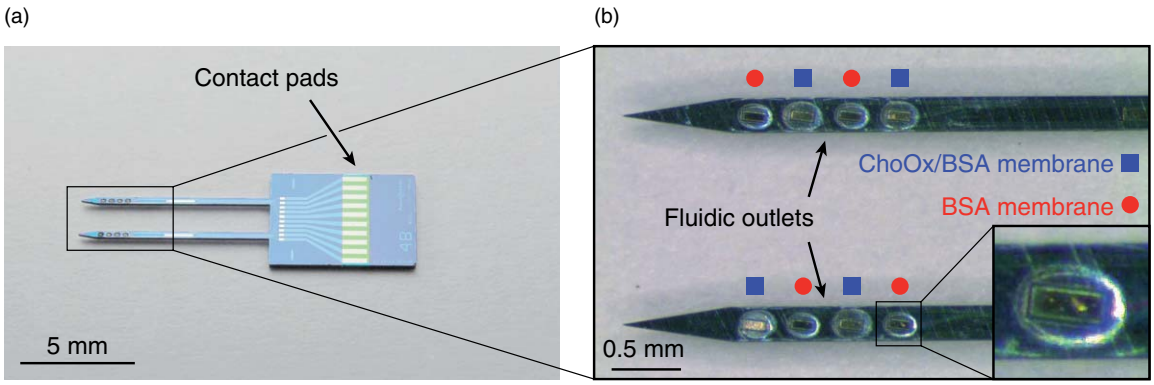

Figure 3. (a) Top view of a microprobe comprising one microfluidic channel and five electrodes per probe shank. The four electrodes near the tip are coated with either an enzyme or a protein membrane. (b) Close-up view of the two microprobe shanks with the membrane-coated electrodes as well as an enlarged view of a single electrode.

compression distance to ensure good electric contact. The assembly is fixed using a custom-made printed circuit board (PCB) screwed to the holder from the top. The PCB comprises metal pads with the same layout as those on microprobe base and a standard electrical connector.

The main advantages of this approach are:

- Fluidic and electrical connections are provided in a rapid, single assembly step. This substantially saves packaging time and costs. Especially for relatively short-living biosensors—being the life-time limiting component of the entire device—this is highly beneficial.
- The packaging is reversible and thus allows us to re-use the holder several times and to store the microprobes separately. Especially the second point strongly facilitates storage and transportation, as in many cases the biosensors have to be kept in a liquid environment at low temperatures (e.g. PBS at 4 °C).
- The whole device is small and fits well into standard stereotaxic frames. Furthermore, its compact size enables experiments with freely behaving animals.

3. Connections

Electric connections are provided by a fitting male connector (Farnell AG, Wallisellen, Switzerland) and a shielded cable to the electronic set-up. For liquid delivery, Portex® fine bore polythene tubing (0.28 mm ID, 0.61 mm OD) connects the steel tubes of the device to a conventional syringe pump.

4. Results and discussion

4.1. Fabrication and packaging

Microprobes have been fabricated according to the described process and showed an excellent reproduction of the designed probe shape and adequate electrochemical properties of the Pt microelectrodes comparable to previous results (Frey *et al* 2010) as well as functional microfluidic channels. A representative photograph of a microprobe is shown in figure 3(a). Prior to the probe assembly into the PC holder, the microprobes are rinsed thoroughly using deionized water and dried using a nitrogen stream. The ZEBRA® connectors and o-rings are wiped or rinsed with isopropanol before use. This ensures a reliable and moisture-free connection. Due to the construction of the device and its self-aligning property, the packaging of the microprobe as shown in figure 2(b) is completed within 2 min. No visual magnification and special microtechnological tools have to be used, significantly simplifying on-site assembly in the course of *in vivo* recording experiments.

4.2. Liquid delivery

Most critical regarding clogging of the microfluidic channels is the functionalization of the microelectrodes at a distance of only 100 μm from the outlet port. Right after coating of the electrodes, all microchannels were thoroughly rinsed for 5 min with deionized water. No clogging or increased flow-resistance was observed due to the functionalization process. Prior to the experiments, the channels were completely filled from the syringe side toward the outlet port with the desired liquid. Care has been taken not to entrap any bubbles except for a single small one intentionally introduced in the transparent polythene tubing used as a control of fluid delivery. No leakage at the connection sites—especially at the o-ring interconnection—was observed for flow-rates up to 5 μl min^{-1}. The total volume between inlet at the stainless steel tubing and the outlet ports on the microprobes was measured to be less than 700 nl. Further representative fluidic characterization of the microprobes with fluid functionality, e.g. their flow resistance or fluidic backflow, is provided by Spieth *et al* (2009).

4.3. Biosensor characteristics

The applied functionalization procedure enables the local deposition of enzymatic membranes on dedicated electrodes in parallel. A photograph of the front section of a microprobe with all recessed Pt microelectrodes coated with a membrane is shown in figure 3(b). In this example, active membranes containing the enzyme ChoOx and inactive membranes comprising BSA only were alternately deposited as indicated by the symbols in figure 3(b). The image shows membranes of

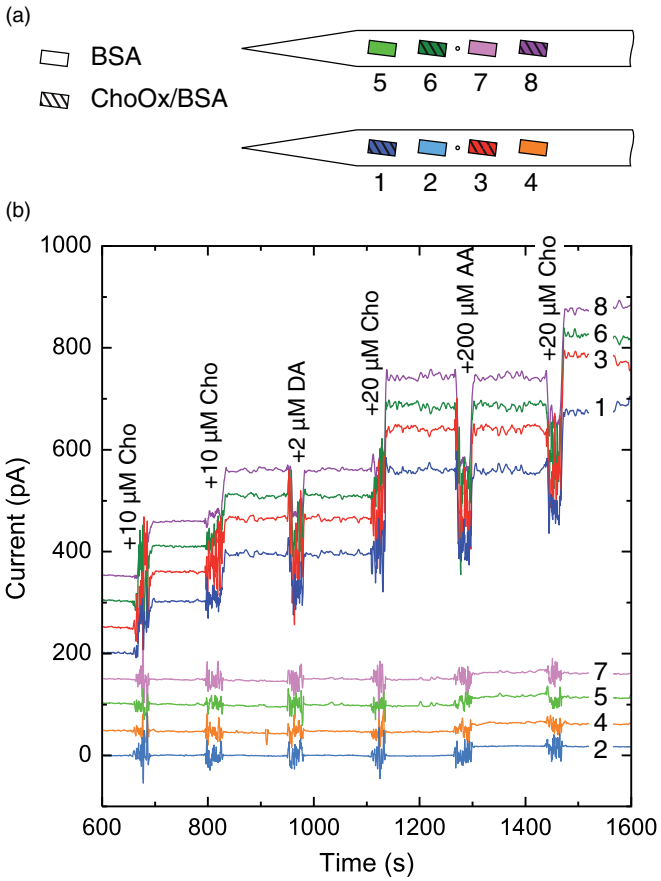

Figure 4. Calibration measurement of a functionalized microprobe. (a) Configuration of the biosensors on the microprobe shanks. (b) Typical response of eight integrated microelectrodes simultaneously recorded upon consecutive additions of choline (Cho) and the two main interferents dopamine (DA) and ascorbic acid (AA) at physiologically relevant concentrations (measurement performed four days after membrane deposition, curves are off-set by 50 pA for better visibility).

reproducible shape on all microelectrodes and confirms that the deposition method is well suited for this kind of microprobe. The recess of the electrodes anchors the membranes on the electrode and prevents them from peeling off during insertion into brain tissue.

The functionalized microprobe was tested in PBS at 37 °C applying a potential of 0.7 V versus Ag/AgCl using a PalmSens potentiostat extended with an 8-channel multiplexer unit (Palm Instruments BV, Houten, The Netherlands) in a three-electrode configuration. A Pt wire served as the counter electrode. Figure 4(b) shows a continuous amperometric measurement of a biosensor microprobe with its eight microelectrodes functionalized according to the schematic shown in figure 4(a). Once the background current reached a stable value, aliquots of choline, dopamine and ascorbic acid were added followed by short stirring (responsible for noise bursts in the measurement curves).

The enzyme-modified electrodes show a rapid step-increase of the current signal upon addition of choline. A response time $t_{90\%}$ of 2 s could be measured. This value, however, is a conservative determination because it is strongly dependent on the mixing time in the calibration solution when the concentration of choline is increased by adding an aliquot in the stirred solution. Dopamine and ascorbic acid on the other hand have no influence on the current signal. This nicely confirms the effective rejection properties of the *m*-PPD blocking layer. Furthermore, all active electrodes exhibit the same sensitivity and selectivity emphasizing the reproducibility of the parallel membrane deposition. The inactive electrodes show no current increases upon choline, DA and AA administration over the entire measurement time. This demonstrates that no cross-contamination during deposition and no cross-talk during the recording occurs between the different closely spaced electrodes. Therefore, they can effectively act as null sensors for the differential measurement. The absence of cross-talk and the continuous and consistent signal from all electrodes further supports good interconnection characteristics of the ZEBRA® connectors. Contact pads are well aligned and all electrodes are independently addressable. The noise-level of the current response is comparable with the wire-bonding techniques previously used for such microprobes (Frey *et al* 2010).

Figure 5 shows the evolution of the biosensor sensitivity and its interference rejection properties in the first month after functionalization. The microprobes ($n = 4$) were disassembled and stored in PBS at 4 °C between the individual experiments. As expected, the sensitivity of the biosensor decreases over time due to partial inactivation of the enzyme ChoOx. At the same time, the *m*-PPD blocking layer becomes slightly permeable to DA and AA. In these cases, the adjacent inactive electrodes showing the same temporal behavior are used to remove the unwanted current contributions applying the differential method (i.e. subtraction of the signal at electrode #2 from signal at electrode #1, figure 4(a)). It should be noted that with our experimental setup, a sensitivity of 3 pA μM^{-1} is still sufficient for reliable *in vivo* measurements with a limit of detection below 1 μM. A 'shelf lifetime' of at least one month offers the flexibility to prepare the biosensor some time in advance of the *in vivo* experiment. Once implanted, the enzymatic membranes are in contact with living tissue at a constant temperature of 37 °C. Proteases and reactive species for example might accelerate the sensitivity decay and may limit the use of the biosensors primarily to acute measurements. The *in vivo* lifetime of the biosensors has not been evaluated for the present system at this stage, but definitively is a crucial point that has to be considered. It is clear that pre- and post-calibration will be required. Further, the integrated channels offer the possibility of an *in vivo* calibration by injection of a known choline concentration or chemical stimulants evoking a known choline release. *In vivo* calibration and its influence on the local brain tissue, however, has to be investigated thoroughly prior to experiments. The local tissue needs to be restored to its initial biological state before reliable measurements can be executed.

4.4. Simultaneous delivery and recording

Both the liquid delivery and the functionality of the biosensors were simultaneously tested in agarose gel (2% in 10 mM PBS)

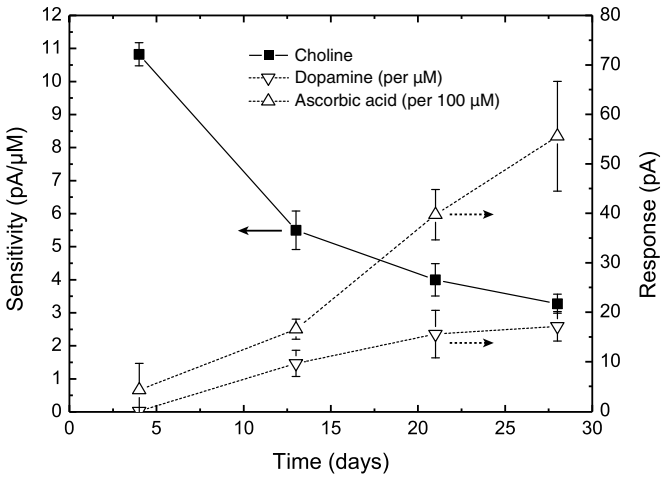

Figure 5. Evolution of the sensitivity of the choline biosensors and current response to dopamine (DA, 1 μM) and ascorbic acid (AA, 100 μM) during the first month after functionalization. The microprobes were disassembled and stored in PBS at 4 °C between individual measurements.

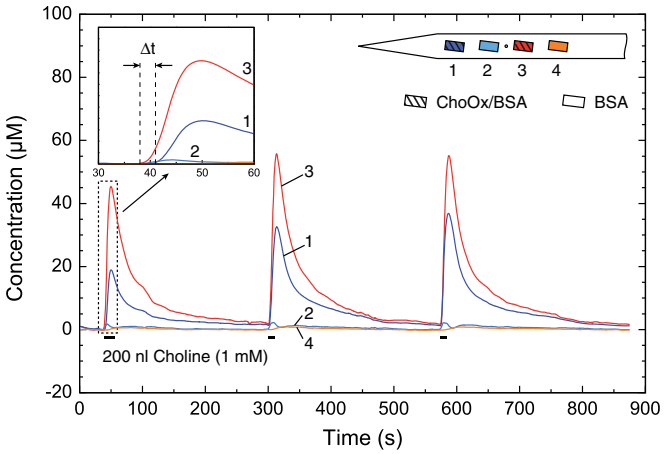

Figure 6. Simultaneous injection and chemical recording from the same probe shank. Choline aliquots (1 mM, 200 nl) are injected into agarose gel using a syringe pump (500 nl min^{-1}) while simultaneously recording from all four electrodes (0.65 V versus Ag/AgCl-wire).

used as a brain phantom (similar to Chen *et al* 2004). The microprobe was vertically inserted into the gel using Ag/AgCl and Pt wires as reference and counter electrodes, respectively. Both wires were inserted at a distance of approximately 10 mm from the biosensor. Subsequently, 200 nl aliquots of 1 mM choline solution were injected through the microchannel of the probe shank while recording from all four electrodes. Figure 6 shows the response of the electrodes comprising membranes with either ChoOx/BSA or BSA only. The biosensor electrodes record an immediate current peak due to the diffusion of choline into the agarose gel, whereas no response is visible on the inactive electrodes. Additionally, electrode #1 located farther away from the microfluidic outlet port, reproducibly shows a lower response shifted by Δt in time compared to electrode #3 positioned next to the outlet port. The time shift Δt corresponds to the slow diffusion of choline in agarose gel. Further experiments characterizing the injection of liquids into agarose gel were performed with microfluidic probes without biosensor as described elsewhere (Spieth *et al* 2009).

5. Conclusions

Recessed biosensor electrodes were integrated on silicon microprobe arrays comprising one or two microfluidic channels per shank for the local delivery of substances at a distance of 100 μm from the electrodes. The functionalization of the electrodes is spatially controlled and can be performed in parallel on several electrodes without clogging of the microfluidic channels. The biosensors show satisfying sensitivity and selectivity for the detection of choline in the first three weeks after fabrication. Functional *in vitro* tests in agarose gel demonstrate simultaneous chemical stimulation and recording. It is clear that the presented response time and size of the biosensor excludes recording of direct synaptic transmission. In our case, the main interest lies in slower concentration changes in larger areas resulting from neurotransmitter 'spill-over' and non-synaptic release. Further, cholinergic neurons are known to innervate most regions of the central nervous system and, for example, the performance on attentional paradigms can lead to large and sustained increases in cortical acetylcholine release, which is subsequently broken down to choline.

Regarding microprobe layout, the shank cross-section of 250 μm $\times$ 250 μm is rather at the upper limit. Smaller dimensions will reduce brain damage but require higher pressures for liquid delivery due to smaller channel dimensions and higher demands on the wafer bonding process to generate leakage-free microchannels. These issues are actually under investigation in a second generation process.

Besides presenting for the first time multisite-biosensing and fluidic delivery functionalities combined in the same microprobe shank, the crucial issue of packaging was addressed in this paper as well. Assembling microfabricated devices into a functional system including all required connectics is most often a time consuming and expensive task. Especially when electric and fluidic connections have to be provided to devices that have a short lifetime, optimized and simple solutions are even more important. The clamping mechanism in a custom-made, compact polycarbonate holder presented herein not only combines electrical as well as fluidic interconnection, but allows us to reversibly package the microprobes just before their application. This significantly facilitates their storage in a liquid environment. Further, the holder can be used several times, i.e. for electrode functionalization, calibration, *in vivo* measurements, etc. Its construction allows precise alignment of the important connection parts without any specialized instruments. In the future, one can think of biosensor functionalization and calibration of multiple microprobes at the same time using an adapted holder with multiple slots based on the same clamping concept. Even a stacking of microprobes toward three-dimensional microelectrode arrays is possible.

Future work includes functional assessment of the microprobes in the medial prefrontal cortex of the rat brain.

Also sterilization schemes prior to implantation and the evaluation of their impact on the biosensor performance need to be addressed. Many groups cited in this paper reported similar systems *in vivo* without stating serious problems regarding this issue. Amongst the possible sterilization schemes, gamma radiation was reported as having no significant influence on the sensor characteristics (Koudelka-Hep *et al* 1993).

The device size is also sufficiently small for recordings in freely behaving animals (e.g., rats). In the latter case, further precautions have to be taken to protect the electric contacts. This can be solved using dissolvable glues such as dental acrylic to fix and insulate the critical components. Moreover, to minimize movement restrictions of the task-performing animal, highly flexible electric and fluidic cables, and eventually commutators depending on the task, are a prerequisite.

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